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DOI: https://doi.org/10.3732/apps.1200515

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Author(s): Hassan Mansour , Ares Jiménez , Barbara Keller , Michael D. Nowak , and Elena Conti
Source: Applications in Plant Sciences, 1(6) 2013.
Published By: Botanical Society of America
DOI: http://dx.doi.org/10.3732/apps.1200515
URL: http://www.bioone.org/doi/full/10.3732/apps.1200515

Applications in Plant Sciences

Applications in Plant Sciences 2013 1(6): 1200515

Primer Note

Development of 13 microsatellite markers in the endangered Sinai primrose (*Primula boveana*, Primulaceae)[1]

Hassan Mansour[2,5], Ares Jiménez[3,4,5], Barbara Keller[3], Michael D. Nowak[3], and Elena Conti[3]

[2]Department of Botany, Faculty of Science, Suez Canal University, Ismailia 41522, Egypt; and [3]Institute of Systematic Botany, University of Zurich, Zollikerstrasse 107, Zurich 8008, Switzerland

- *Premise of the study:* We developed microsatellite markers for the endangered plant *Primula boveana*, the Sinai primrose, and assessed the cross-transferability of these markers to six related taxa.
- *Methods and Results:* DNA sequences containing microsatellites were isolated from a microsatellite-enriched library. We obtained successful amplification of 13 microsatellite primer pairs, seven of which were polymorphic in *P. boveana.* Eleven of these primers successfully cross-amplified to related taxa.
- *Conclusions:* The markers reported herein will be useful to characterize the genetic diversity of the endangered *P. boveana* and to evaluate its mating system, and have the potential to be useful for similar studies in close relatives.

Key words: cross-amplification; *Dionysia*; microsatellites; *Primula boveana*; Sinai; sect. *Sphondylia.*

The Sinai primrose, *Primula boveana* Decne. ex Duby (Primulaceae), has been reported as one of the rarest and most endangered plant species worldwide (Richards, 2003). It is endemic to Mount St. Catherine, in the Sinai mountains in southern Egypt, where it has been located in only five clearly delimited localities at least one kilometer from each other, all of them consisting of fewer than 10 to a few hundred adult plants. This species, restricted to wadis (i.e., valleys of intermittent streams) fed by meltwater near the top of Mount St. Catherine, is severely threatened by both natural and human factors. The most important natural threats are the fragmentation inherent to its habitat and the aridity of the area, with very scarce precipitation year round. Human impacts, especially water collection for human consumption, sheep and goat grazing, and traditional plant collection for medicinal uses, further intensify the natural threats of aridification and fragmentation, thus pushing *P. boveana* to the brink of extinction.

Primula boveana belongs to sect. *Sphondylia* (Duby) Rupr., which, together with its sister group, the genus *Dionysia* Fenzl, forms a well-supported clade within *Primula* L. (Mast et al., 2001, 2006). All the species included in sect. *Sphondylia*, as well as some *Dionysia* species, are rare, narrow endemics distributed in wet refugia in arid areas from northeastern Africa to Southwest Asia. Because of the rarity of these species, genetic diversity and mating system studies are needed to warrant the conservation of these taxa. Here, we report 13 microsatellite loci that will be used to characterize the genetic diversity and mating system of *P. boveana*, and test their cross-amplification with three other *Primula* species belonging to sect. *Sphondylia* and with three *Dionysia* species.

METHODS AND RESULTS

DNA isolated from our specimen AS35 of *P. boveana* from the population in Ain Shennarah (see below) was used by Genetic Marker Services (Brighton, United Kingdom; http://www.geneticmarkerservices.com) to develop a microsatellite-enriched library and to design and test microsatellite primer pairs. Enrichment involved incubating adapter-ligated, size-restricted DNA with filter-bonded synthetic repeat motifs, $(AG)_{17}$, $(AC)_{17}$, $(AAC)_{10}$, $(CCG)_{10}$, $(CTG)_{10}$, and $(AAT)_{10}$. Thirty-nine positive library colonies were selected for sequencing, from which 22 microsatellites were designed and tested for amplification. The primer pairs were designed using the software Primer3 version 3.0 (Rozen and Skaletsky, 2000), with the criterion of amplifying products in the range of 100–250 bp to minimize later overlap ambiguities during multiplexing genotyping projects. We tested each primer pair for amplification and polymorphism in eight individuals of *P. boveana* that represented four of the five populations in Mount St. Catherine: Ain Shennarah (28°31′N, 33°57′E; $N = 2$), wadi Shaq Mousa (28°31′N, 33°57′E; $N = 2$), wadi Gebal (28°33′N, 33°52′E; $N = 2$), and Kahf El-Ghoula (28°32′N, 33°56′E; $N = 2$). The 13 primer pairs that resulted in amplification products in *P. boveana* (Table 1) were further tested for cross-amplification in one individual each of several closely related *Primula* and *Dionysia* species (Table 2). Representative voucher specimens for every taxon are deposited in herbariums E (Royal Botanic Garden Edinburgh), SCU (Suez Canal University), and Z (University of Zurich; Appendix 1).

Prior to DNA extraction, ~20 mg of dry leaf tissue per individual was ground with stainless steel beads using an MM 3000 shaker (Retsch GmbH, Haan, Germany). Total genomic DNA was extracted using the DNeasy Plant Mini Kit (QIAGEN, Hombrechtikon, Switzerland) following the manufacturer's guidelines. Amplification of microsatellite loci was performed following the

[1]Manuscript received 27 September 2012; revision accepted 16 November 2012.

The authors thank M. Meloni for her valuable advice on protocols to screen for microsatellite polymorphisms and S. Hussein for his help in sampling *Primula boveana* in the field. This study was supported by the Swiss National Science Foundation grant IZK0Z3_139418 to H.M. and E.C. and by the University of Zurich.

[4]Author for correspondence: ares.jimenez@gmail.com

[5]These authors contributed equally to this work.

doi:10.3732/apps.1200515

TABLE 1. Characterization of 13 microsatellite loci isolated from *Primula boveana*.

Locus	GenBank accession no.	Repeat motif	Size range (bp)	Primer sequences (5′–3′)	*A*	H_o	H_e
Prim45a	JX154138	$(CT)_9$	200	F: CAGAGTCACAGTCTTGTAGCTT R: CACACACACACACAGAGACCA	1	—	—
Prim45b	JX154138	$(TG)_{12}$	197–203	F: GCACTACAGTAACCTCAAAAGG R: TCCCAATCTGCTGTATTGTC	3	0	0.046
Prim48	JX154139	$(TCA)_6$	161	F: GGAACCAATTCGCTGAACC R: CGGATGATGATGAGGAGGAG	1	—	—
Prim49b	JX154155	$(GT)_{12}$	186–188	F: GTGTGTGGTGGTGGTGGTAA R: AGGTGAATCCAAAATGCAAA	2	0	0.032
Prim53	JX154159	$(GA)_{11}$	218	F: GACTCACAGGACCGGGACTA R: AGAGGTTGGGATAGCGGTTC	1	—	—
Prim54	JX154160	$(GA)_{16}$	166–168	F: AAAGGCGGGAAGAAAATGTTA R: CCCAGAAGGAGAGAATGAGAA	2	0	0.074
Prim58	JX154142	$(AC)_6AA(AC)_6$	104	F: CGTCTTTGAAACCATTCCTTG R: CACACATCTCTCCCCCTCTC	1	—	—
Prim59	JX154143	$(AG)_{10}$	200–216	F: GCAACATGAACCATTGCTTG R: GGAAGAAAAACGGGTTACGA	4	0	0.036
Prim61b	JX154167	$(TG)_{22}$	206–228	F: GTGTGTGTGTGTGTGTATGGAC R: AAACCTGCAAAACTCCTGCT	3	0.031	0.127
Prim62b	JX154168	$(AC)_9$	128	F: GCGTTAGCGGACTAATAGCA R: CATGAGCTCCTTTCCGACAC	1	—	—
Prim65	JX154149	$(GA)_{11}$	198	F: AGCAGGAGCACTACCAACAAA R: CCCTCATCCCGATTTCTTC	1	—	—
Prim64	JX154148	$(AG)_{12}$	251–254	F: CGATCAAAACCAACAAAACCC R: GATCAAACATGCTAATGCTGCT	2	0	0.144
Prim66	JX154150	$(AC)_9$	146–148	F: TCTCCCTCCCTTTTACTCTTCC R: TGGGCTAACATGGAAGGTTG	2	0.038	0.138

Note: — = monomorphic locus; *A* = number of alleles per locus; H_e = expected heterozygosity; H_o = observed heterozygosity.

single-reaction, nested PCR method of Schuelke (2000), a cost-efficient method best suited for projects with a small to moderate number of samples (Blacket et al., 2012). PCRs were performed in a final volume of 25 µL containing 2.5 µL of 10× reaction buffer, 1 µL of $MgCl_2$ (50 mM), 0.5 µL of a mix of all four dNTPs (10 mM), 0.2 µL of the forward primer incorporating the M13-tail (10 µM; Schuelke 2000), 0.5 µL of the reverse primer (10 µM), 0.5 µL of the universal M13 primer (10 µM; Schuelke, 2000) labeled with a fluorophore (FAM, NED, VIC, or PET), 0.1 µL of *Taq* DNA polymerase (Bioline GmbH, Luckenwalde, Germany; 50 U/µL), 1.0 µL of bovine serum albumin (BSA; 20 mg/mL), 1.0 µL of 10 ng/µL genomic DNA, and sterilized water up to the final volume. All PCRs were carried out in singleplexes using a T1 Thermocycler (Biometra GmbH, Göttingen, Germany) under the following conditions: initial denaturation at 94°C for 3 min; 30 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 1 min; eight cycles of 94°C for 30 s, 53°C for 45 s, and 72°C for 1 min; and a final extension step of 72°C for 5 min. The resulting fluorescently labeled PCR products were run in multiplexes on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, California, USA) using GeneScan 500 LIZ Size Standard (Applied Biosystems) as a size standard and scored using GeneMapper 4.1 (Applied Biosystems), following the recommendations given by Arif et al. (2010).

Seven of the 13 microsatellite primers amplified polymorphic products in *P. boveana* (Table 1). Genetic diversity parameters and deviations from Hardy–Weinberg equilibrium were estimated using GenAlEx version 6.4 (Peakall and Smouse, 2006) on 20 individuals randomly sampled from each of three populations (Ain Shennarah, wadi Shaq Mousa, and wadi Gebal) for a total of 60 individuals. The number of alleles observed for the seven polymorphic loci ranged from two to four, and the observed and expected heterozygosities ranged from 0 to 0.038 and from 0.032 to 0.144, respectively (Table 1). In the three populations studied, all of the observed genotype frequencies of loci with more than one allele significantly departed from the expectations of Hardy–Weinberg equilibrium ($P < 0.001$), with the exception of locus Prim61 in the wadi Gebal population.

Eleven primer pairs amplified PCR products in at least one of the three other *Primula* and three *Dionysia* species tested (Table 2). Accordingly with the expectations of higher cross-transferability of microsatellites to the taxa more closely related to the focal species (e.g., Primmer et al., 1996), the success of cross-amplification was higher in the other *Primula* species of sect. *Sphondylia* (11 out of 13 primers resulted in amplification) than in *Dionysia* (eight out of 13 primers resulted in amplification).

CONCLUSIONS

The set of seven polymorphic loci out of the 13 microsatellites reported here is adequate to further investigate the mating system and population genetic structure of *P. boveana*. More specifically, measurements of genetic diversity and estimations of selfing and outcrossing rates will be used to understand the evolutionary responses of the mating system of *P. boveana* to the factors threatening its persistence in the wild. Furthermore, the six microsatellites reported here as monomorphic may still provide useful genetic information if they are polymorphic in other populations of *P. boveana*. Additionally, the successful cross-amplification of 11 microsatellite loci to other species of *Primula* sect. *Sphondylia* and of genus *Dionysia* open up the possibility, provided that they amplify polymorphic products, of studying the genetic variation of other endangered taxa in this group of plants.

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Table 2. Cross-amplification of *Primula boveana* microsatellites to three other *Primula* species from sect. *Sphondylia* and to three *Dionysia* species.[a]

Locus	*P. edelbergii*	*P. floribunda*	*P. simensis*	*D. gaubae*	*D. hedgei*	*D. tapetodes*
Prim45a	231 233	233 235	223 225	131 133	231 233	130 132
Prim45b	203	—	—	—	—	—
Prim48	+	—	+	+	—	+
Prim49b	+	—	178	+	116	—
Prim53	219	—	237	—	—	—
Prim54	153	—	169	+	+	+
Prim58	+	+	122	+	—	+
Prim59	+	—	218	187 191	—	+
Prim61b	197	+	195	+	+	+
Prim62b	122	—	141	—	—	—
Prim64	265 267	258	249	+	—	+
Prim65	—	—	—	—	—	—
Prim66	—	—	—	—	—	—

Note: + = amplification of not readily interpretable products requiring further optimization; — = no amplification.
[a]All cross-amplifications were tested on a single individual per species. Numbers represent allele size (in base pairs).

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Appendix 1. Information on voucher specimens for taxa included in this study. Vouchers are deposited in herbaria E (Royal Botanic Gardens Edinburgh), Z (University of Zurich), and SCU (Suez Canal University).

Taxon	Voucher specimen (Herbarium)	Geographic origin (Geographical coordinates)	Distribution range
Primula boveana Decne. ex Duby	S. A. Gamal El-din 340 (SCU)	University of Suez Canal, Ismailia, Egypt; ex Mount St. Catherine, Egypt (28°31′N, 33°57′E)	Egypt
P. edelbergii O. Schwarz	A. R. Mast 715 (Z)	University of Newcastle upon Tyne, Newcastle, England; ex Götteborg Botanic Garden	Afghanistan
P. floribunda Wall.	A. R. Mast 714 (Z)	University of Newcastle upon Tyne, Newcastle, England; ex Royal Botanic Gardens Edinburgh, Edinburgh, Scotland	Afghanistan, Pakistan, India, Nepal
P. simensis Hochst.	A. R. Mast 712 (Z)	University of Newcastle upon Tyne, Newcastle, England; source of plant uncertain	Ethiopia, Somalia
Dionysia gaubae Bornm.	F. Ghahremani-nejad 135 (Z)	Lorestan, Iran (33°23′N, 47°58′E)	Iran
D. hedgei Wendelbo	D. S. Feller 34113 (Z)	Mazar-I Sharif, Afghanistan (36°43′N, 67°05′E)	Afghanistan
D. tapetodes Bunge	CULTE 15012 (E)	Royal Botanic Gardens Edinburgh, Edinburgh, Scotland; ex Baghlan, Afghanistan: 18 miles E of Banu, S of the village of Pul-i-Sar	Afghanistan, Iran